

Cell-Specific Regulatory Modules Control Expression of Genes in Vascular and Visceral Smooth Muscle Tissues

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Abstract—A novel approach with chimeric SM22 α /telokin promoters was used to identify gene regulatory modules that are required for regulating the expression of genes in distinct smooth muscle tissues. Conventional deletion or mutation analysis of promoters does not readily distinguish regulatory elements that are required for basal gene expression from those required for expression in specific smooth muscle tissues. In the present study, the mouse telokin gene was isolated, and a 370-bp (–190 to 180) minimal promoter was identified that directs visceral smooth muscle–specific expression in vivo in transgenic mice. The visceral smooth muscle–specific expression of the telokin promoter transgene is in marked contrast to the reported arterial smooth muscle–specific expression of a 536-bp minimal SM22 α (–475 to 61) promoter transgene. To begin to identify regulatory elements that are responsible for the distinct tissue-specific expression of these promoters, a chimeric promoter in which a 172-bp SM22 α gene fragment (–288 to –116) was fused to the minimal telokin promoter was generated and characterized. The –288 to –116 SM22 α gene fragment significantly increased telokin promoter activity in vascular smooth muscle cells in vitro and in vivo. Conversely, a fragment of the telokin promoter (–94 to –49) increased the activity of the SM22 α promoter in visceral smooth muscle cells of the bladder. Together, these data demonstrate that both vascular- and visceral smooth muscle–specific regulatory modules direct gene expression in subsets of smooth muscle tissues. (*Circ Res.* 2002;91:1151-1159.)

Key Words: SM22 α ■ telokin ■ gene regulation

Smooth muscle cells arise from diverse populations of precursor cells during embryonic development, and the mechanisms that specify the smooth muscle cell phenotype in each of these populations of cells are largely unknown. All differentiated smooth muscle is characterized by the presence of unique isoforms of contractile proteins (such as smooth muscle α - and γ -actin, myosin heavy chain, caldesmon, SM22 α , telokin, and calponin) that are not expressed in other tissue types. Analysis of the spatial and temporal pattern of expression of several smooth muscle proteins has revealed distinct patterns of expression of these proteins in different smooth muscle tissues during development.^{1–6} This suggests that it is likely that distinct and overlapping mechanisms control the expression of genes in different smooth muscle tissues. The heterogeneity of regulatory mechanisms likely reflects the diverse embryological origins of smooth muscle cells in these different tissues.

The *cis*-acting regulatory elements of the SM22 α , telokin, smooth muscle myosin heavy chain, smooth muscle α - and γ -actin, and desmin genes have been shown to direct reporter gene expression to smooth muscle tissues in transgenic mice.^{7–14} However, each of these promoters mediates a distinct pattern of transgene expression. For example a 2.4-kb fragment of the rabbit telokin promoter directs high levels of transgene expression in smooth muscles of the gut, airways,

and reproductive and urinary tracts and low levels of expression in vascular smooth muscle. This pattern of expression mirrors the expression of endogenous telokin.^{5,15} A 16-kb fragment of the smooth muscle myosin gene, including 4.3 kb of the proximal promoter and the first intron, directs expression to all smooth muscle tissues except the pulmonary vasculature.⁸ In contrast, truncated myosin gene fragments direct different patterns of transgene expression depending on the precise deletions made.^{9,12} The pattern of expression of these transgenes in various smooth muscle tissues suggests that distinct regulatory elements or modules, as well as distinct transcription factors, are required for the expression of a single gene in different smooth muscle tissues. This is further supported by results obtained from analysis of the SM22 α promoter in transgenic mice. A 441-bp SM22 α promoter is sufficient to direct expression to arterial but not venous or visceral smooth muscle tissues, although the endogenous SM22 α is expressed in all smooth muscle tissues. This suggests that additional regulatory elements may be required for expression in venous and visceral smooth muscle compared with arterial smooth muscle.^{13,14,16–18}

Analysis of SM22 α gene regulatory elements in transgenic mice has also suggested that different regulatory paradigms may operate during embryonic and postnatal development. DNase I footprint analysis of the minimal artery-specific

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441-bp SM22 α promoter fragment has revealed the presence of six protein-binding regions, designated SME1 to SME6.¹³ Multimerized copies of either SME1 or SME4 are sufficient to direct arterial expression of either a minimal SM22 α promoter or the heat shock protein 68 promoter^{16,17} in mice at embryonic day 11.5. However, these elements were not sufficient to restrict the expression to arterial smooth muscle in postnatal animals.¹⁶

In the present study, we demonstrate that the addition of a 172-bp fragment from the SM22 α gene to the telokin promoter increases telokin promoter activity in vascular smooth muscle in adult mice. In contrast to the native SM22 α promoter, the chimeric 172SM22 α /telokin promoter was active in both arterial and venous smooth muscle as well as in visceral smooth muscle tissues. In a reciprocal experiment, the addition of a 45-bp fragment of the telokin gene to the SM22 α promoter increased the activity of the SM22 α promoter in bladder smooth muscle cells. Together, these data demonstrate that modular transcription elements control the expression of genes in different smooth muscle tissues.

Materials and Methods

Genomic Library Screening

A 129/SvJ mouse genomic DNA library in Lambda ZAP (Stratagene) was screened using standard procedures with a probe that encompassed the 5' end of the mouse telokin cDNA.⁵

Reporter Constructs and Transgenic Mouse Production

All promoter luciferase-reporter genes were constructed as described previously.¹⁵ For the generation of transgenic mice, promoter fragments indicated in the figure legends were cloned into the AUG-LAC transgene cassette.^{7,14} Detailed methods on reporter gene and transgene construction and analysis are provided in the online data supplement (available at <http://www.circresaha.org>).

Results

Identification and Characterization of the Mouse Telokin Promoter

A 21-kb genomic clone was isolated that encompasses the 3' portion of the MLCK-telokin gene, including the entire telokin coding and promoter region. *EcoRI* fragments (1.7 and 6.1 kb) of this clone that include the 5' regulatory region and first two exons of telokin were sequenced and used for generating reporter genes (GenBank accession No. AF542075). A blast search of the mouse genome revealed that the sequence of this clone is contained within a supercontig on mouse chromosome 16 (GenBank accession No. NW_000107). The translation start site of telokin is located at bp 31 795 887 of this contig. Sequence alignment of the mouse telokin promoter with the rabbit¹⁹ and human²⁰ telokin sequences revealed a high degree of sequence homology between nucleotides -213 and +67 (80% identity; please see online data supplement). All of the regulatory elements previously identified in the rabbit promoter are conserved in the mouse and human promoters.

A Fragment (370 bp) of the Mouse Telokin Promoter Is Sufficient to Mediate Cell-Specific Expression of Telokin Promoter In Vitro

Fragments of the mouse telokin promoter indicated in Figure 1A were fused to a luciferase reporter gene and

transfected into A10 vascular smooth muscle cells and REF52 fibroblasts. Results of reporter gene assays show that a 361-bp fragment of the mouse promoter (-190 to 171) is sufficient to mediate maximal smooth muscle cell-specific expression of a reporter gene. Additional

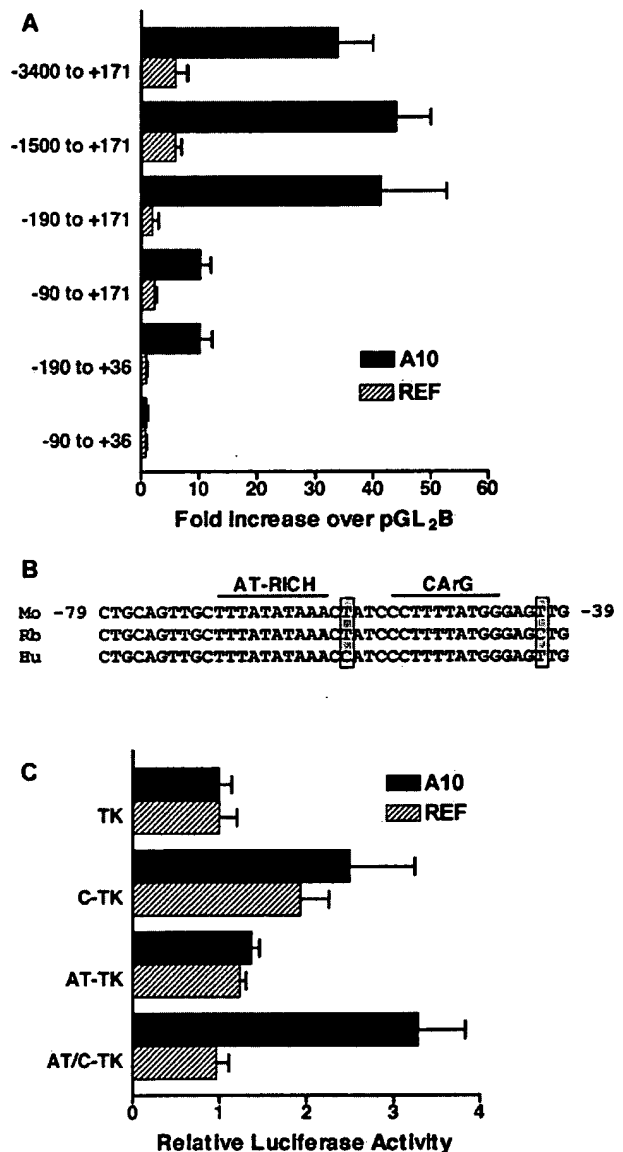


Figure 1. Luciferase activity directed by fragments of the mouse telokin promoter. **A**, Relative luciferase activities (mean \pm SEM of 6 samples) obtained from the fragments of the mouse telokin promoter indicated at the left of the graph are shown. Luciferase fusion genes were transfected into A10 and REF52 (REF) cells, and luciferase activity was measured. Luciferase activities were normalized to a β -galactosidase internal control and are expressed as fold stimulation over a promoterless luciferase vector (pGL2B). **B**, Sequence alignment of the AT-CArG region of the mouse, rabbit, and human telokin promoters is shown. **C**, Two copies of the telokin AT-rich region (AT), CArG motif (C), or an AT-rich region + CArG motif (AT/C) were placed 5' to the minimal thymidine kinase (TK) promoter in the sense orientation. Luciferase fusion genes were transfected into A10 and REF cells, and luciferase activity was measured. Luciferase activities (mean \pm SEM of 6 assays) expressed as a percentage of the activity obtained from the minimal TK promoter alone in A10 cells and normalized to a β -galactosidase internal control are shown.

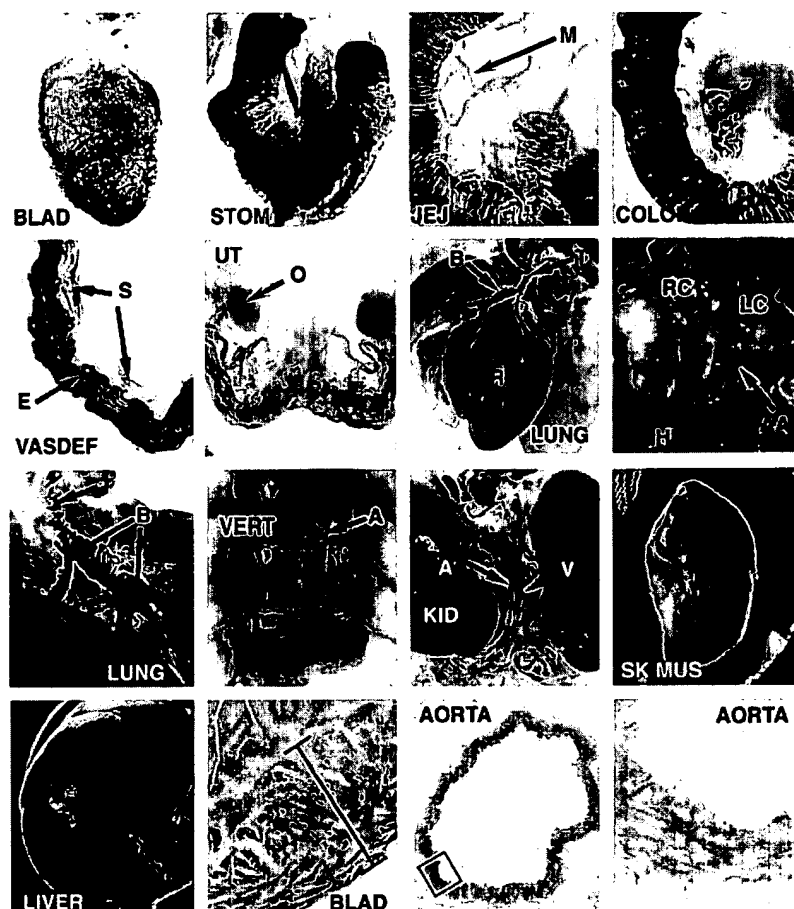


Figure 2. wtTel370-LAC transgene expression in an adult mouse. Whole tissues from an adult 370-bp (–190 to 180) telokin promoter transgenic mouse were stained for β -galactosidase activity and then photographed or cleared with methyl salicylate before photography, as described in Materials and Methods. Images shown were obtained from one animal, which is representative of the 4 lines characterized as having detectable β -galactosidase expression. Dark blue coloring corresponds to β -galactosidase activity. Tissues are indicated as follows: BLAD indicates bladder; STOM, stomach; JEJ, jejunum; VASDEF, vas deferens; UT, uterus; HRT, heart; VERT, vertebrae; KID, kidney; and SK MUS, skeletal muscle. Specific features are indicated as follows: M, mesenteric vasculature; S, smooth muscle cells; B, bronchi; T, trachea; O, ovary; RC, right carotid; LC, left carotid; AA, aortic arch; A, aorta; and V, vena cava. Nonspecific staining of epithelial cell lining of the VASDEF is indicated (E). BLAD and aorta from another animal from this same transgenic line were cryosectioned, stained for β -galactosidase activity, and counterstained with hematoxylin and eosin. A bracket designates the smooth muscle layer of the BLAD, and the boxed region of the low-magnification aorta is magnified in the adjacent picture.

deletions to nucleotide –90 reduced reporter gene activity in A10 cells; however, the resulting truncated promoter still directed 5-fold greater expression in A10 cells compared with REF52 cells (Figure 1A).

The AT-Rich Region, Together With the Adjacent CArG Box, Acts as a Smooth Muscle-Specific Regulatory Module In Vitro

We have previously shown that a CArG box and the adjacent AT-rich region (Figure 1B) are important for telokin promoter activity.²¹ To evaluate the importance of these regions in mediating cell-selective promoter activity, dimers of the CArG box, AT-rich region, or both were fused 5' to a minimal thymidine kinase promoter reporter gene, and their activity was analyzed in A10 and REF52 cells (Figure 1C). The AT-rich region alone was found to have little effect on the activity of the thymidine kinase promoter; in contrast, as reported previously, the CArG box alone increased promoter activity \approx 2-fold in both A10 and REF52 cells.²¹ Together, the AT-rich region and CArG box increased thymidine kinase promoter activity 3-fold in A10 cells without significantly increasing promoter activity in REF52 cells (Figure 1C). These data demonstrate that the CArG box in the telokin promoter appears to collaborate with an adjacent AT-rich region in mediating cell-specific expression of the promoter.

The Telokin Promoter Mediates Smooth Muscle-Specific Expression of a Transgene In Vivo in Mice

We have previously shown that both 2.4-kb and 310-bp fragments of the rabbit telokin promoter direct smooth muscle-specific expression of a simian virus 40 large-T-antigen transgene in adult transgenic mice.^{15,22} On the basis of the similarity between the rabbit and mouse sequences and in vitro activity of their promoters, it was anticipated that a 370-bp minimal mouse telokin promoter would direct smooth muscle-specific β -galactosidase activity in vivo in transgenic mice. To confirm this hypothesis, wtTel370-LAC transgenic mice, in which a 370-bp mouse telokin promoter (–190 to 180) was used to drive expression of a β -galactosidase transgene, were generated. Expression of β -galactosidase directed by the telokin promoter was then analyzed in F1 neonatal and adult animals and adult F0 founder mice of each independent transgenic line. A total of 14 lines were generated, and of these, only four lines expressed detectable levels of β -galactosidase. The inability to detect β -galactosidase expression in many of the founder lines may reflect the relative insensitivity of the LacZ assay. Alternatively, because the level of expression was not copy number dependent (data not shown), the site of transgene integration may be influencing expression, such that expression is only detectable if the transgene integrates into a region with open

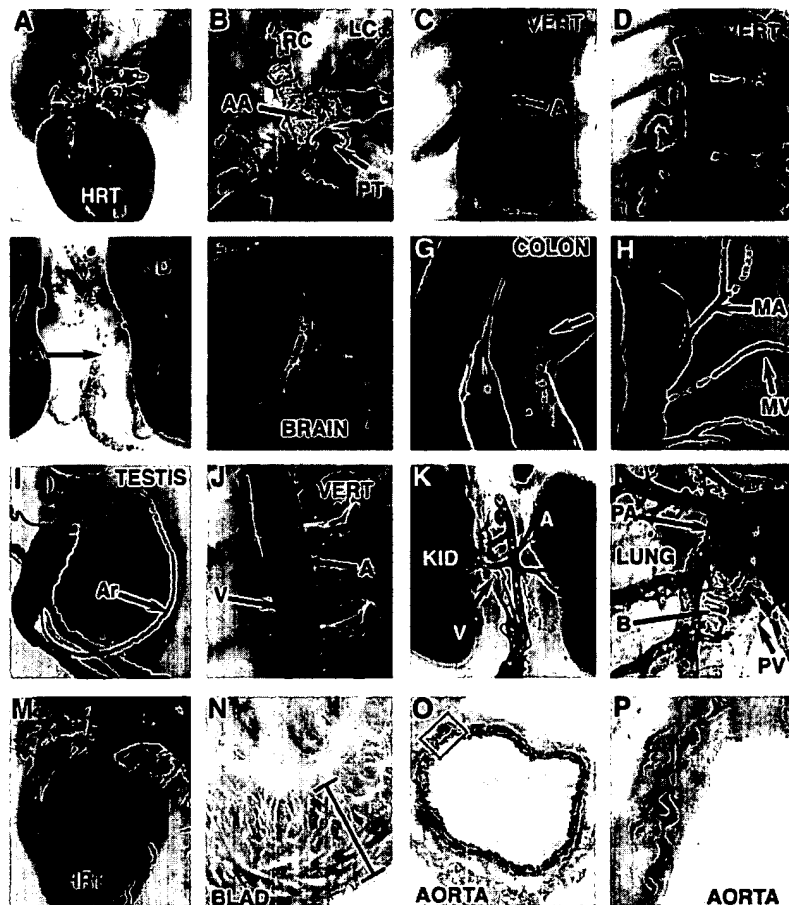


Figure 3. wtSM22 α -LAC transgene expression in adult mice. Whole tissues from adult mice of 3 different wtSM22 α (–475 to 61) promoter transgenic lines were stained for β -galactosidase activity as described in Materials and Methods. Panels A, B, C, E, and N are images of tissues from one transgenic mouse line; panels D, H, O, and P are from another line; and panels F, G, I, J, K, L, and M are from a third line. Blue coloring corresponds to β -galactosidase activity. Arrows point to specific features, such as the RC, LC, AA, pulmonary trunk (PT), aorta (A), mesenteric vein (MV), mesenteric artery (MA), artery (Ar), bronchi (B), pulmonary artery (PA), pulmonary vein (PV), coronary artery (C), and vena cava (V). Cryosections of BLAD and aorta were stained for β -galactosidase activity and then counterstained with hematoxylin and eosin (panels N through P). A bracket designates the smooth muscle layer of the BLAD (N), and the boxed region of the aorta (O) is magnified in the adjacent section (P).

chromatin structure. It might be anticipated that the endogenous telokin promoter is within such a relaxed and transcriptionally favorable region, because the promoter lies within the myosin light chain kinase gene, a gene that is ubiquitously expressed in all cell types.¹⁹

β -Galactosidase expression in wtTel370-LAC transgenic mice was largely restricted to visceral smooth muscle tissues in neonatal and adult mice, with high levels of expression throughout the digestive system and bladder as well as the reproductive tract, trachea, and bronchi (Figure 2). β -Galactosidase was rarely, if ever, detected in large or small vessels of the vascular system. Expression was absent from skeletal and cardiac muscle tissues and nonmuscle tissues, such as the liver and kidney, although one of the lines displayed ectopic neuronal expression (data not shown). These results demonstrate that a 370-bp fragment of the mouse telokin promoter is sufficient to mediate transgene expression to adult visceral but not vascular smooth muscle *in vivo*. In contrast, this transgene appeared to be expressed more broadly and variably in day-11.5 to -14.5 embryos (data not shown). β -Galactosidase-stained cryosections verified that the expression was restricted to smooth muscle cells of the muscularis layer of the bladder and gut (Figure 2). However, expression in smooth muscle tissues was observed to be heterogeneous with some but not all cells staining positively for β -galactosidase. This may reflect episodic transcription from the telokin promoter, as previously described for the smooth muscle myosin promoter.²³

An SM22 α Transgene Targets Arterial Smooth Muscle Tissues in Neonatal and Adult Mice

Expression of SM22 α transgenes has been extensively studied during embryonic development, but transgene expression in postnatal animals has not been as well characterized.^{13,14,18} Therefore, we have examined the expression of the SM22 α promoter (–475 to 61) in adult transgenic mice in more detail. Of nine wtSM22 α transgenic lines that transmitted and expressed β -galactosidase, one had no detectable adult expression, although strong embryonic expression was present at day 14.5 (data not shown). The eight remaining wtSM22 α -LAC lines expressed significant but variable levels of β -galactosidase in arterial smooth muscle cells (Figure 3). This variability was independent of both the level of embryonic arterial expression and the transgene copy number, which varied from 1 to 10. Arterial expression extended from the aortic arch to the abdominal aorta (Figures 3A to 3E). Consistent staining of the small arteries of the cerebral vasculature was also observed in all eight lines (Figure 3F), but small arteries associated with the skeletal muscles or mesentery exhibited variable staining (Figures 3G to 3I). In the three lines with the most robust arterial β -galactosidase staining, low levels of staining were also detected in portions of the venous and pulmonary vasculature (Figures 3J to 3L), and two of these three also had detectable expression in the coronary arteries (Figure 3M). A few positive visceral smooth muscle cells were also scattered in the gut, bladder, or bronchi

of each of these three lines with robust arterial β -galactosidase staining (Figure 3L). In addition to β -galactosidase expression in smooth muscle cells, three lines of wtSM22 α -LAC mice also expressed β -galactosidase in cardiac muscle cells. Two of these had light staining in the right atria and a portion of the right ventricle (eg, Figure 3A), whereas the other line had intense staining throughout the heart (data not shown). The levels of expression in cardiac muscle did not correlate with the levels of expression in vascular smooth muscle. β -Galactosidase-stained cryosections of the thoracic aorta and bladder demonstrated that SM22 α expression is restricted to smooth muscle cells of the aorta and is not observed in the muscularis layer of the bladder (Figures 3N to 3P). Taken together, these results generally confirm previous findings that the SM22 α promoter targets transgene expression to arterial smooth muscle cells in the adult. However, 3 of the 8 wtSM22 α -LAC transgenic lines also exhibiting significant venous staining (although very little staining of visceral smooth muscle cells) were observed.

Addition of an SM22 α Gene Fragment to the Telokin Promoter Increases Promoter Activity in A10 Vascular Smooth Muscle Cells

An SM22 α promoter fragment extending from -475 to 61 bp was fused to a luciferase reporter gene and analyzed in A10 vascular smooth muscle cells and REF52 rat embryo fibroblasts. In agreement with previous *in vitro* data, this promoter was highly active in both rat smooth muscle and nonmuscle cells.²⁴ Compared with the mouse telokin promoter, the SM22 α promoter exhibited 7-fold greater activity in A10 vascular smooth muscle cells and 25-fold greater activity in REF52 fibroblasts (Figure 4A).

Fusion of a -475 to -110 SM22 α gene fragment to the -190 to +171 telokin promoter stimulated telokin promoter activity 6-fold in A10 vascular smooth muscle cells and 2.5-fold in REF52 fibroblasts (Figure 4A). Alone, the -475 to -110 SM22 α fragment exhibited no significant promoter activity. To determine whether the -475 to -110 SM22 α gene fragment acts as an enhancer, its position and orientation dependence were evaluated (Figure 4B). Results show that reversing the orientation of the -475 to -110 SM22 α fragment or moving it more distal to the promoter abolished its ability to enhance telokin promoter activity.

To further delineate the regulatory region within the -475 to -110 SM22 α gene fragment, additional chimeric promoters were generated that constituted either the proximal (-288 to -110) or distal (-475 to -289) half of this fragment (Figure 4A). Analysis of these chimeric promoters revealed that the proximal SM22 α gene fragment (-288 to -110) contained all of the activity of the larger fragment, whereas the distal -475 to -289 fragment of the SM22 α gene was unable to enhance telokin promoter activity.

An SM22 α /Telokin Chimeric Promoter Directs Transgene Expression to Vascular and Visceral Smooth Muscle Tissues *In Vivo*

Results described above predict that the -288 to -110 SM22 α fragment constitutes a vascular smooth muscle-

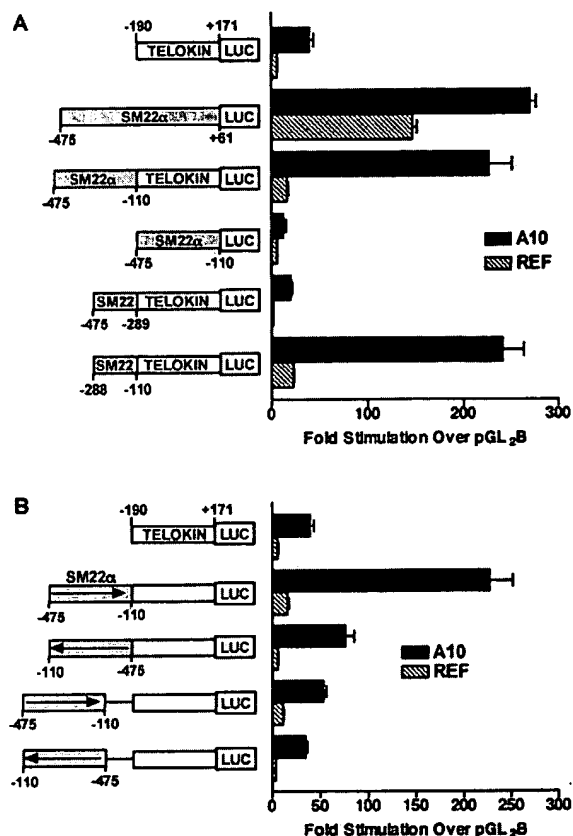


Figure 4. Analysis of chimeric promoters *in vitro*. A, Promoter-luciferase fusion genes shown at the left of the figure were transfected into A10 and REF cells, and luciferase activity was measured. The same minimal telokin promoter was used in each of the constructs indicated. Luciferase activities (mean \pm SEM of 4 to 10 assays) expressed as fold stimulation over a promoterless vector and normalized to a β -galactosidase internal control are shown. B, Orientation and position dependence of the -475 to -110 SM22 α gene fragment were determined by reversing its orientation (indicated by the direction of the arrows on the SM22 fragment, indicated by the shaded box) or by placing it in a site at the opposite side of the pGL₂B vector (the additional space between the fragment and the promoter is indicated by the thin line), as indicated at the left of the graph. Constructs were analyzed as described in panel A.

specific regulatory module. To test this prediction, we analyzed the β -galactosidase expression in 172SM22 α /telokin promoter (SM22 -288 to -116/telokin -190 to +180)-LAC transgenic mice (Figure 5). Of the 19 172SM22 α /telokin lines generated, 17 displayed β -galactosidase expression in arterial smooth muscle. In 15 of these lines, β -galactosidase activity, identified by histochemical staining, was also observed in visceral smooth muscle of the digestive, urogenital, and respiratory tracts (Figure 5). In 9 of these 17 positive 172SM22 α /telokin-LAC lines, β -galactosidase expression was also detected in the venous smooth muscle associated with the vena cava, pulmonary vasculature, or the mesenteric veins (Figure 5). No β -galactosidase activity was detected in the coronary vasculature of any line. Expression levels in the heart were variable among lines but, overall, were higher than those observed in transgenic mice in which β -galactosidase activity was driven by either the SM22 α or telokin promoter

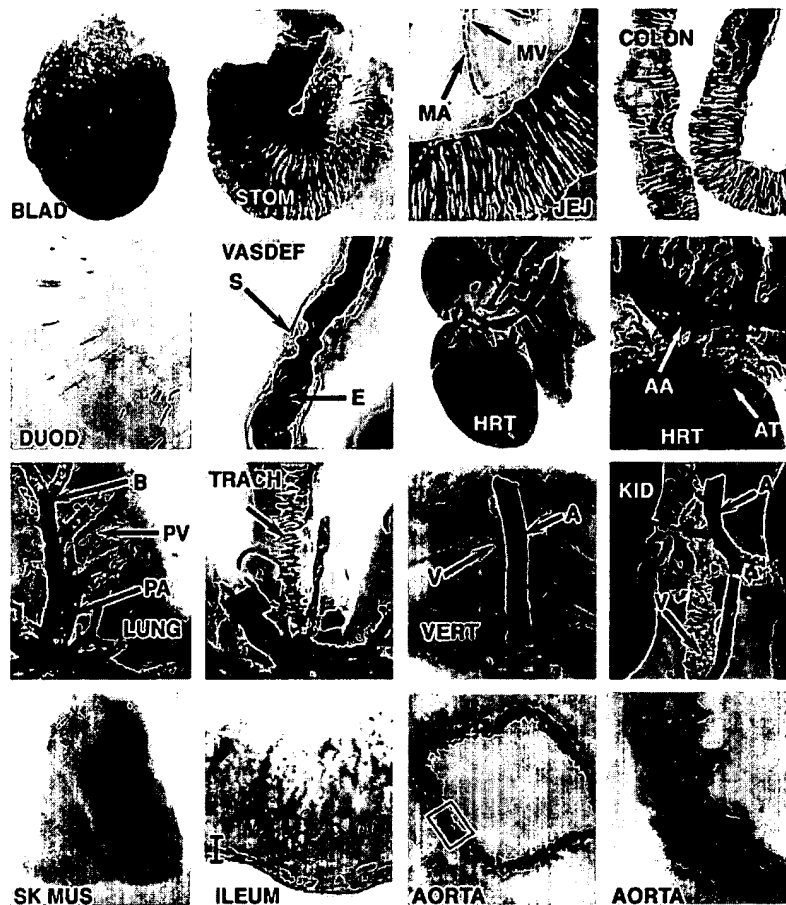


Figure 5. 172SM22 α /telokin-LAC transgene expression in a neonatal mouse. Whole tissues from an adult mouse harboring an SM22 α (–288 to 116)/telokin(–190 to 180) chimeric promoter-LAC transgene were stained for β -galactosidase activity, cleared with methyl salicylate, and photographed as described in Materials and Methods. Images obtained from 1 of the 9 172SM22 α /telokin-LAC transgenic lines with significant expression in venous smooth muscle are shown. DUOD indicates duodenum; TRACH, trachea. Arrows point to specific features such as the mesenteric artery (MA), mesenteric vein (MV), smooth muscle cells (S), AA, atria (AT), PA, PV, bronchi (B), aorta (A), and vena cava (V). Nonspecific staining of the epithelial cell layer of the VASDEF is also indicated (E). Cryosections of ileum and aorta from another representative animal from the same transgenic line were stained for β -galactosidase activity and counterstained with hematoxylin and eosin. A bracket designates the smooth muscle layer of the ileum, and the boxed region of the low-magnification section of the aorta is magnified in the adjacent panel.

alone (Figures 2 and 3). Cryosections of the thoracic aorta and gut show that β -galactosidase activity is specifically targeted to the smooth muscle cells of tissues but that it is present in both the vascular and visceral smooth muscle cells (Figure 5). As previously observed with the SM22 α promoter transgene, β -galactosidase expression was consistently expressed in the cerebral arteries on the ventral surface of the brain in 172SM22 α /telokin chimeric promoter transgenic lines but not in the telokin transgenic lines lacking the SM22 α gene fragment (Figure 6). Analysis of the transcription start sites used by the chimeric promoter transgenes, using 5' RACE analysis, confirmed that the chimeric promoter used the same transcription start sites as the endogenous telokin gene (data not shown).

A Telokin AT-CaRG/SM22 α Chimeric Promoter Directs Transgene Expression to Visceral and Vascular Smooth Muscle Tissues In Vivo

To test the hypothesis that the AT-rich/CaRG region of the telokin promoter may include a visceral smooth muscle-specific regulatory module, this region of the telokin promoter was fused to the 536-bp SM22 α promoter (–475 to +61), and its ability to increase SM22 α promoter activity in visceral smooth muscle cells was evaluated. Of the 10 AT-CaRG/SM22 α promoter transgenic lines that transmitted and expressed β -galactosidase activity, all had some visceral and vascular smooth muscle staining (Figures 6 through 8). The visceral expression of the

AT-CaRG/SM22 α transgene lines was consistently much lower than that observed in the wild-type telokin promoter or the 172SM22/telokin chimeric promoter transgenic lines, with most of the visceral expression being restricted to the bladder (Figures 7 and 8). In contrast, the arterial staining of the AT-CaRG/SM22 α transgenic lines was comparable to that observed with both the wild-type SM22 α or the 172SM22/telokin transgenic lines (Figures 6 and 7). β -Galactosidase-stained cryosections demonstrated that promoter activity was restricted to the smooth muscle cells of both the bladder and the aorta (Figure 7). These data suggest that at the level of sensitivity of the LacZ assay, the AT-CaRG element from the telokin promoter constitutes a regulatory module that is specific for bladder smooth muscle cells.

Discussion

Results from our analysis of chimeric promoters composed of mouse SM22 α and telokin gene fragments demonstrate that separate gene regulatory modules direct the expression of proteins to specific smooth muscle tissues. A –288 to –116 SM22 α gene fragment contains a gene regulatory module that directs high levels of gene expression to the arterial vasculature. In contrast, a –94 to –49 telokin promoter fragment contains a regulatory module that is able to direct gene expression to visceral smooth muscle of the bladder. In addition, a chimeric promoter composed of the arterial element from the SM22 α gene fused 5' to the visceral smooth muscle-specific minimal

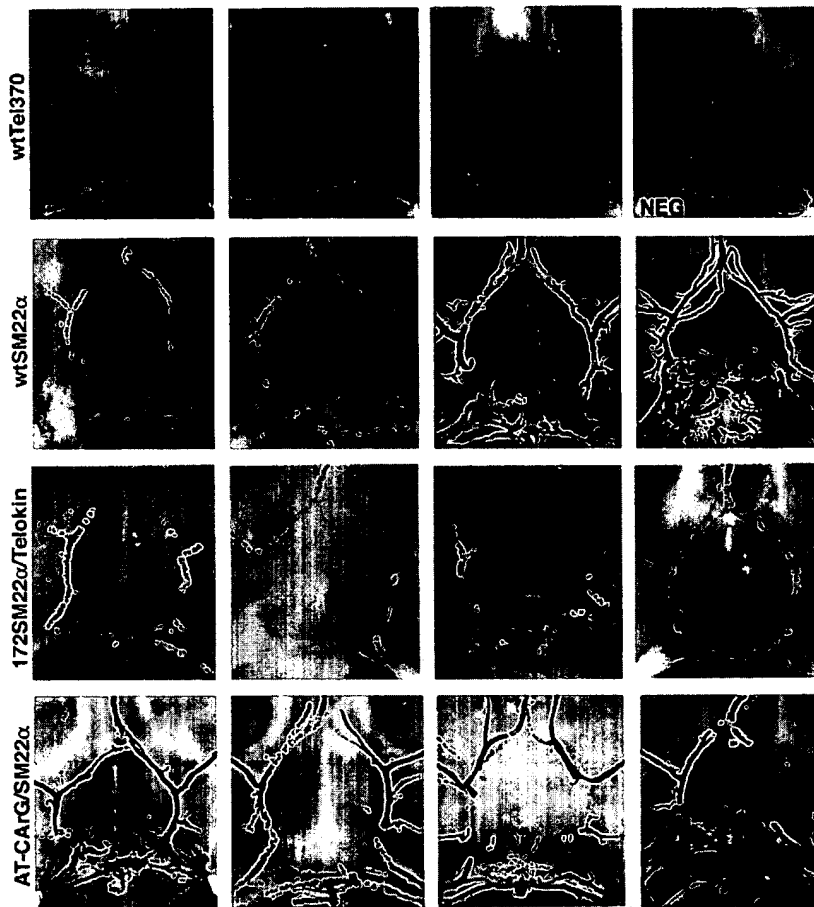


Figure 6. Transgene expression in brain. Whole brains isolated from 3 different wtTel370-LAC transgenic lines, 4 different wtSM22 α -LAC transgenic lines, 172SM22 α /telokin-LAC chimeric transgenic lines, and AT/CArG-SM22 α -LAC chimeric transgenic lines, and one nontransgenic DBA/2 animal (NEG) were stained for β -galactosidase activity as described in Materials and Methods. Blue staining can be seen in the arteries located on the ventral surface of the brain of the wtSM22 α , 172SM22 α /telokin, and AT-CArG/SM22 α transgenic lines but not the wtTel370 transgenic lines or the nontransgenic control brain.

telokin promoter directed transgene expression in venous smooth muscle cells.

We have previously shown that serum response factor binding to a CArG box at -65 to -56 of the telokin promoter is required for promoter activity *in vitro* in smooth muscle cells.²¹ Results from the analysis of reporter genes containing either the CArG box and/or the adjacent AT-rich region fused to a minimal thymidine kinase promoter suggest that the AT-rich region and CArG box may cooperate to activate transcription in a cell-specific manner (Figure 1C). This proposal is supported by data showing that the telokin AT/CArG region increases SM22 α promoter activity in smooth muscle of the bladder. In contrast, this fragment did not significantly increase SM22 α promoter activity in the gut, suggesting that intestinal and bladder smooth muscle cells use distinct gene regulatory elements to direct the expression of telokin and perhaps other smooth muscle proteins. Consistent with this proposal, a region between $+2.5$ and $+11.6$ kb of the smooth muscle myosin gene is required for high levels of promoter activity in urinary but not intestinal smooth muscle cells.¹²

Although previous studies have described arterial restricted expression of transgenes driven by the -475 to $+61$ SM22 α promoter, our data show that in a significant number of transgenic lines, low levels of expression can also be seen in adult venous smooth muscles. The most likely reason for this discrepancy is that we have analyzed expression in postnatal animals from a large number of independent transgenic lines, whereas

most previous studies have focused on embryonic expression, with adult expression being characterized in a limited number of transgenic lines. Because we observed venous expression in only 3 of 17 of our wtSM22 α -LAC transgenic lines, it is also possible that this expression is due to the activity of ectopic enhancers. However, because this pattern was observed in three lines, it is unlikely to be the result of integration next to venous smooth muscle-specific enhancers; it is more likely that this indicates that many enhancers or chromosomal locations can increase the expression of the SM22 α promoter in venous smooth muscle cells. Consistent with this hypothesis, we noted significantly increased β -galactosidase expression in venous smooth muscle in more than half of our transgenic lines in which the -288 to -116 SM22 α gene fragment was fused to the telokin promoter (Figure 5). Chromatin structure at sites of transgene integration and methylation of gene regulatory regions may also result in variable expression of transgenes. In support of this conjecture, when the SM22 α promoter is used to direct LacZ expression in an adenoviral vector, high levels of β -galactosidase expression in vascular and visceral smooth muscle tissues can be obtained.²⁵ In addition, cytosine methylation of the SM22 α (-475 to $+61$) promoter has been reported to downregulate promoter activity *in vitro*.²⁶ Thus, it is possible that variable promoter methylation or chromatin structure, at the site of transgene integration, in different wtSM22 α -LAC transgenic lines may account for the differences in transgene expression in venous smooth muscle in these lines.

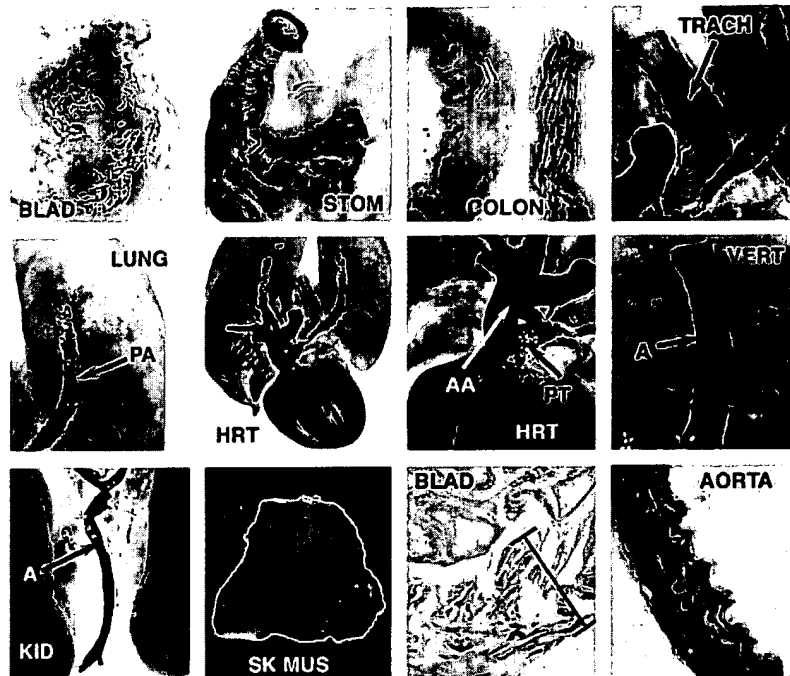


Figure 7. AT-CaRG/SM22 α -LAC transgene expression in an adult mouse. Whole tissues obtained from an adult AT-CaRG (–94 to –49) telokin/SM22 α (–475 to 61) chimeric promoter transgenic mouse were stained for β -galactosidase activity as described in Materials and Methods. Ten independent lines were analyzed; images obtained from one representative animal are shown. Arrows point to specific features, such as the PA, AA, PT, and aorta (A). The pale blue/green staining of the STOM and gut represents nonspecific staining; the darker stripes of blue stain in these tissues are the specific staining of individual smooth muscle cells. Cryosections of BLAD and aorta, from another representative animal from the same transgenic line, were stained for β -galactosidase activity and counterstained with hematoxylin and eosin. A bracket designates the smooth muscle layer of the BLAD, and the boxed region of the low-magnification aorta section is magnified in the adjacent panel.



Figure 8. Transgene expression in bladder. Whole bladders isolated from 3 different wtTel370-LAC transgenic lines, 4 different wtSM22 α -LAC transgenic lines, 172SM22 α /telokin-LAC transgenic lines, and AT/CaRG-SM22 α -LAC transgenic lines, and one nontransgenic DBA/2 line (NEG) were stained for β -galactosidase activity as described in Materials and Methods. β -Galactosidase staining of smooth muscle cells in the bladder was evident in all of the wtTel370, 172SM22 α /Tel, and AT-CaRG/SM22 α lines shown. In contrast, only 1 of the 4 wtSM22 α lines exhibited any positive staining of smooth muscle cells. The pale green color at the interior of the bladders is nonspecific staining.

The 172-bp SM22 α gene fragment used in the present study includes transcription factor binding sites SME1 to SME4 that were previously identified by DNase footprinting experiments.¹³ Two of the elements, SME1 and SME4, contain CARG boxes, and serum response factor binding to these CARG boxes has been shown to be critical for promoter activity.¹⁷ In addition, multimerized copies of either SME1 or SME4 in front of a minimal SM22 α promoter or a minimal heat shock protein 68 promoter directed transgene expression to arterial smooth muscle cells in mouse embryos. However, these transgenes were not sufficient to restrict expression to arterial smooth muscle in adult animals.¹⁶ In contrast, we found that a single copy of a 172-bp SM22 α fragment is sufficient to increase telokin promoter activity specifically in vascular smooth muscle in adult mice. These data suggest that distinct regulatory elements may be required to control smooth muscle-specific gene expression at different stages of development and that specific interactions between regulatory elements within the SM22 α gene may be required to mediate its arterial specificity.

The expression of chimeric SM22 α /telokin promoters in both vascular and visceral smooth muscle tissues also demonstrates the feasibility of generating small artificial promoters that will target gene expression to specific smooth muscle tissues. These results, in combination with previous results from analysis of adenoviruses containing chimeric promoters,²⁷ suggest that by combining cell-restricted regulatory modules, it should be possible to generate designer promoters that would have important clinical applications for directing the expression of therapeutic proteins to specific smooth muscle tissues. However, once generated, these promoters must be carefully tested, because juxtaposing two different modules may not necessarily result in the simple sum of the activities of each module, and interactions between modules may result in new patterns of expression.

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